Immunophenotyping of CD8+ Tscm cells

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Introduction

CD8+ cytotoxic T cells play an important role in clearing out infected or malignant cells. The cytotoxic T cell subsets of central memory (Tcm), effector (Teff), effector memory (Tem), naïve (Tnaïve) and stem cell memory cells (Tscm) are all essential cell types for disease resistance. Tnaïve cells differentiate into memory T cell subsets and effector cells when exposed to an antigen. Some of the memory cells become long-lived Tscm cells which can differentiate when re-exposed. Tscm cells have an increased capacity for self-renewal and are of great interest in vaccine development and cell therapies. Therefore, identification and qualification of CD8+ cytotoxic T cell populations in human whole blood by flow cytometry is critical in a clinical setting as they are used for assessing safety and efficacy in drug development. In this study, qualification of CD8+ cytotoxic T cells was performed using a fit-for purpose approach to overcome the challenges of a phenotypic biomarker assay validation for detecting the rare Tscm events.

Objective

To qualify a flow cytometry method for the determination of CD8+ Cytotoxic T-cell Immunophenotyping of Tscm, Tcm, Teff, Tem and Tnaïve subsets in human whole blood by performing

- Precision (Intra- Assay Repeatability)
- Inter-Instrument Comparison
- Sensitivity, Lower Limit of Detection (LLOD)
- Pre-Process Stability
- Post-Process Stability

Methods

The analytical method can be briefly described as follows (Figure 1): Fresh human whole blood (EDTA) is stained with a 6-color antibody cocktail (Table 1) designed to enumerate CD8+ cytotoxic T-cell subsets (Tscm, Tcm, Teff, Tem and Tnaïve).

Figure 1: Sample Processing Procedure

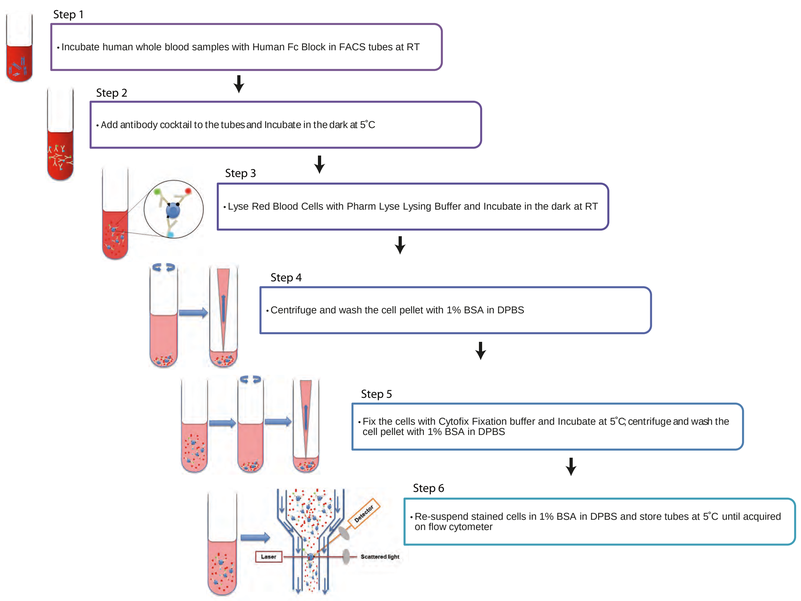

Table 1: Antibody Panel

Laser	Fluorochrome	Marker
Blue 488	FITC PE PerCP CY 5.5 PE-Cy7	CD62L CD95 CD45RA CD3
Red 633	Alexa 647 APC-H7	CD197 (CCR7) CD8

Results

Figure 2: Gating Strategy
Gating Strategy used for detecting different CD8+ cytotoxic T-cell subsets (Tscm, Tcm, Teff, Tem and Tnaïve). Lymphocyte population was gated on FSC/SSC plot (A). Lymphocytes were then further gated to remove doublets (B) and determine CD3+ CD8+ cells (C). CD3+ CD8+ cells were then gated to define Tcm, Teff, Tem and Tnaïve subsets (D) and CD45RA+ CCR7+ cells (E). CD95 and CD62L were used on CD45RA+ CCR7+ gate to determine Tscm cells.

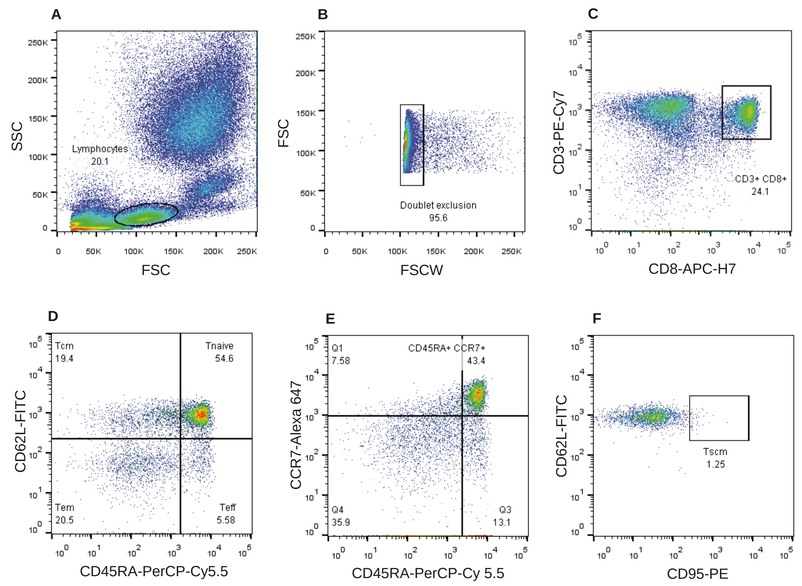

Table 2: Precision (Intra- Assay Repeatability)
Six different subjects analyzed in triplicate were run by 2 different analysts (3 subjects/ analyst) on 2 different days.

Sample ID	Mean CD3+CD8+	Mean Tscm	Tscm% of CD3+CD8+	Mean Tcm	Tcm% of CD3+CD8+	Mean Teff	Teff % of CD3+CD8+	Mean Tem	Tem% of CD3+CD8+	Mean Tnaïve	Tnaïve% of CD3+CD8+
1451	8306	134	1.6	1297	15.6	592	7.1	3379	40.8	3039	36.5
SD			0.1		0.6		0.3		3.3		4.0
% CV			3.5		3.7		4.9		8.0		11.0
1452	7636	85	1.1	1629	21.3	916	12.0	2354	30.9	2737	35.8
SD			0.1		1.1		0.5		3.0		4.5
% CV			6.7		5.1		4.5		9.7		12.5
1453	7761	169	2.2	1187	15.3	787	10.2	1753	22.6	4034	51.9
SD			0.2		0.2		0.8		1.7		2.6
% CV			9.3		1.5		8.1		7.7		5.0
1468	8316	195	2.3	1712	20.6	566	6.8	1925	23.2	4113	49.5
SD			0.2		1.4		0.5		0.5		1.3
% CV			7.6		6.9		7.2		2.3		2.7
1469	6913	67	1.0	851	12.3	441	6.4	1406	20.5	4215	60.8
SD			0.1		0.4		1.2		3.6		5.2
% CV			8.3		3.6		18.8		17.6		8.6
1470	8369	87	1.0	2412	28.8	247	2.9	2119	25.3	3591	42.9
SD			0.0		1.0		0.2		0.8		1.4
% CV			2.0		3.5		6.6		3.0		3.3

Table 3: Inter-Instrument Comparison
Six replicates from one subject were evaluated on 2 different instruments with the same configuration.

Instrument ID	Mean CD3+CD8+	Mean Tscm	Tscm% of CD3+CD8+	Mean Tcm	Tcm% of CD3+CD8+	Mean Teff	Teff% of CD3+CD8+	Mean Tem	Tem% of CD3+CD8+	Mean Tnaïve	Tnaïve% of CD3+CD8+
1	7670	102	1.3	1267	16.5	941	12.3	2144	27.9	3318	43.3
SD			0.1		0.9		0.3		1.1		1.0
% CV			8.5		5.6		2.0		4.1		2.4
4	8249	104	1.3	1246	15.1	1033	12.5	2651	32.1	3320	40.2
SD			0.1		0.4		0.4		1.1		0.9
% CV			7.6		2.8		3.5		3.5		2.1
% Difference			5.1		8.8		2.0		13.9		7.3

Table 4: Sensitivity, Lower Limit of Detection (LLOD)
Three different test subjects were analyzed in triplicate, excluding the CD95 antibody, to determine background contribution to the Tscm subset values.

Sample ID	Mean CD3+CD8+	Mean Tscm	Tscm% of CD3+CD8+	Mean Tcm	Tcm% of CD3+CD8+	Mean Teff	Teff% of CD3+CD8+	Mean Tem	Tem% of CD3+CD8+	Mean Tnaïve	Tnaïve% of CD3+CD8+
1468	7222	11	0.2	1320	18.3	391	5.4	1653	22.9	3858	53.4
1469	4247	14	0.3	540	12.7	287	6.8	1061	25.1	2359	55.4
1470	6848	14	0.2	1723	25.2	289	4.2	1438	21.0	3398	49.6
Overall Mean			0.2								
Overall SD			0.1								
LLOD			0.5								

Table 5: Pre-Process Stability
Three different test subjects samples were maintained in vacuum blood collection tubes at RT for up to 8 hours after collection prior to processing in triplicate.

Time (Hour)	Sample ID	Mean CD3+CD8+	Mean Tscm	Tscm% of CD3+CD8+	Mean Tcm	Tcm% of CD3+CD8+	Mean Teff	Teff% of CD3+CD8+	Mean Tem	Tem% of CD3+CD8+	Mean Tnaïve	Tnaïve% of CD3+CD8+
1	1468	8316	195	2.34	1712	20.6	566	6.8	1925	23.2	4113	49.5
	1469	6913	67	0.96	851	12.3	441	6.4	1406	20.3	4215	61.0
	1470	8369	87	1.04	2412	28.8	247	2.9	2119	25.3	3591	42.9
8	1468	8536	205	2.40	1753	20.5	487	5.7	1809	21.2	4486	52.6
	1469	7531	85	1.13	560	7.4	268	3.6	1035	13.7	5667	75.3
	1470	8275	95	1.14	2320	28.0	196	2.4	1864	22.5	3895	47.1
% of Control	1468			102		100		84		92		106
(1 Hour)	1469			117		60		56		68		123
	1470			110		97		80		89		110

Table 6: Post-Process Stability
Three different test subjects samples were maintained in polystyrene FACS tubes at 5°C protected from light for up to 4 hours after processing.

Time (Hour)	Sample ID	Mean CD3+CD8+	Mean Tscm	Tscm% of CD3+CD8+	Mean Tcm	Tcm% of CD3+CD8+	Mean Teff	Teff% of CD3+CD8+	Mean Tem	Tem% of CD3+CD8+	Mean Tnaïve	Tnaïve% of CD3+CD8+
0	1543	11882	103	0.87	1656	13.9	1732	14.6	4171	35.1	4323	36.4
	1544	11025	98	0.89	2359	21.4	1196	10.9	3038	27.6	4432	40.2
	1545	8266	109	1.32	592	7.2	1912	23.1	931	11.3	4831	58.4
4	1543	11457	97	0.84	1900	16.6	1620	14.1	4162	36.3	3775	33.0
	1544	8064	64	0.79	1885	23.4	802	9.9	2352	29.2	3025	37.5
	*1545	4782	43	0.89	478	10.0	1215	25.4	662	13.8	2428	50.8
% of Control	1543			97		119		97		103		91
(0 Hour)	1544			89		109		92		106		93
	1545			67		139		110		123		87

*Mean calculated from 2 replicates. 3rd replicate lost in processing.

Conclusion

A method has been developed and qualified for the CD8+ Cytotoxic T-cell Immunophenotyping. The initial gating of the lymphocyte population in the FSC/SSC plot and then the CD3+ CD8+ population allows the cytotoxic T cell subsets to emerge. Although the Tscm subset is a rather small population, gating on the CD45RA+, CCR7+ and CD95+ cells allows for a clear presentation of them. The summary of the qualification components are as follows:

- Precision (Intra- Assay Repeatability), %CV for each CD8+ subset (Tcm, Teff, Tem and Tnaïve) was ≤ 20.0% and for Tscm was ≤ 25.0%.
- Inter-Instrument Comparison showed % difference to be ≤20% for each subset
- Sensitivity, Lower Limit of Detection (LLOD) of Tscm was determined to be 0.5% of CD8+ cytotoxic T-cells
- Pre-Process Stability was established for up to 8 hours after collection when stored in vacuum blood collection tubes at ambient temperature.
- Post-Process Stability was established for up to 4 hours after processing when stored in polystyrene FACS tubes at 5°C protected from light.

References

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